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A NEW STAIN FOR BACTERIAL CAPSULES WITH SPECIAL REFERENCE TO PNEUMOCOCCI.*

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The methods used for demonstrating the capsule of pneumococci and other bacteria differ greatly, yet all convey the idea that the capsule is extremely perishable, difficult to fix, and very soluble in water.

In a study of autolysis of pneumococci in NaCl and other solutions the simple staining methods of Welch,[1] Hiss,[2] Boni,[3] Buerger,[4] and others were found unsatisfactory in determining whether the capsule or the coccus is first to disintegrate. The differential methods of Wadsworth[5] and Buerger,[6] while more useful, were also found unreliable in this respect.

After much experimentation the following method has been devised. It has proven of such great value in a study of autolysis of pneumococci, in their identification in culture and exudates as well as in staining the capsule of *Streptococcus mucosus* and, with a slight modification, the capsule of *B. mucosus* also, that a brief report seems desirable at this time.

* Received for publication January 15, 1911.

1 *Johns Hopkins Hosp. Bull.*, 1892, 13, p. 128.

2 *Jour. Exp. Med.*, 1904, 6, p. 335.

3 *Münch. med. Wchnschr.*, 1900, 47, p. 1262.

4 *Centralbl. f. Bakt.*, 1, Orig., 1905, 39, pp. 216, 335.

5 *Jour. Infect. Dis.*, 1906, 3, p. 610.

6 *Jour. Infect. Dis.*, 1907, 4, p. 426

Directions.—Make a thin smear on a perfectly clean slide or cover-glass. If the exudate, such as sputum, is too thick, add enough distilled water so that the material can be spread evenly by means of a piece of fine tissue or cigarette paper. In case of cultures (blood agar, serum—glucose agar or Loeffler's blood serum being preferable) remove a small amount of the growth from the surface of the medium and at once mix thoroughly with a loop-full of serum on the slide and spread by means of tissue paper, or, better still, make a rather dense suspension in a few drops of distilled water, and then mix an equal quantity of this suspension with serum. As the smear becomes nearly dry, cover for 10 to 20 seconds with a 5 to 10 per cent aqueous solution of tannic acid; wash in water and blot; stain with carbol- (saturated alcoholic solution gentian violet [Grübler] 1 pt., 5 per cent phenol in water, 4 pts.) or anilin-gentian-violet $\frac{1}{2}$ to 1 minute, heat over flame but do not boil; wash in water again; Gram's iodine solution for $\frac{1}{2}$ to 1 minute; decolorize in alcohol (95 per cent); stain for from 2 to 10 seconds, depending upon the thickness of smears, with saturated alcoholic (60 per cent) solution of Grübler's eosin; wash in water and blot; finally, clear in xylol and mount in balsam, or examine directly. (If the organism like *B. mucosus* is Gram-negative it may be stained with Loeffler's or aqueous methylene blue.)

The pneumococci are stained deeply brownish-black, sharply differentiated from the capsule, which is stained pink. Beautiful results are also obtained with the *Streptococcus mucosus*. In the thickest part of the smear the space occupied by the capsule may be perfectly clear; elsewhere in the smear, if properly made, where the conditions are suitable for absorption of eosin, the capsule is stained deeply pink; not rarely a clear retraction zone (often mistaken for the capsule in former methods) may be seen peripherally to a distinctly stained, often large, capsule.

In case of sputum in which the cocci are imbedded in a more or less tenacious mucus the capsules, at times, are not rendered stainable by the above method. In that case it is well to fix and stain simultaneously with the 2 per cent aqueous tannic acid, 4 parts, and saturated solution of gentian violet, 1 part. This modification often gives beautiful results. The cocci, however, decolorize

easily and the tannic-acid-gentian-violet may be followed by carbol-gentian-violet and then the usual procedure. Ordinary carbol-fuchsin diluted 5 to 10 times, aqueous eosin (50 per cent sat. sol.) may also be used to stain the capsule although the saturated alcoholic (60 per cent) eosin has given the best results. Decolorization after the modified Gram procedure of tannic-acid-fixed smears is more rapid than in the case of heat-fixed smears, which should be borne in mind.

The age and the completeness of saturation of the alcoholic solution of gentian violet is an important factor. Old "ripened" solutions give the best results. An alcoholic solution of gentian violet from an old stock bottle in which a considerable insoluble residue was present gave uniformly good results without the use of tannic or other acids. Smears fixed in heat, saturated aqueous solution of $HgCl_2$, Mueller's or Zenker's fluid, and less constantly formalin-fixed smears gave beautiful capsules with this stain. Most of the drawings and microphotographs were made from these specimens. Freshly prepared solutions were found unsatisfactory unless the smears were treated with an acid. The changes which take place in the ripening process in these solutions would seem to be the development of acidity, because fresh solutions act like old solutions on adding tannic or other acids and on first treating the smear by various acids, all of which, if used in the proper concentration, increase the affinity of the capsule for acid dyes provided the smear is subsequently treated with a strong basic dye such as carbol-gentian-violet or methyl violet. Just as the acids increase the affinity of the capsule for eosin, so they diminish (but all not to the same degree) the power of the coccus to retain the basic stain. The acids, in the order of efficiency, which have been tested in this way are tannic (2–10 per cent aqueous solutions), phosphomolybdic, phosphotungstic, picric (in various solutions), glacial acetic, and hydrochloric ($N/20$).

While the action of these acids is similar, their efficiency in rendering the capsule stainable, but, at the same time, not rendering the coccus Gram-negative, varies greatly. Tannic acid has been found superior to the others, especially if the smear is not previously fixed by heat or other methods. Phosphomolybdic acid

ranks second and gives excellent preparations in exudate as Smith[1] has recently shown, but is unsatisfactory for cultures of pneumococci because the coccus is rendered too easily Gram-negative.

L. Pelet-Jolivet[2] and others have shown that most staining reactions are colloidal phenomena. That the staining of the capsules of pneumococci is of this nature is indicated by the fact that the smears from cultures need to be made in an albuminous (colloidal) fluid such as serum or egg-white and that treatment in the colloidal solution of carbol- or anilin-gentian-violet or methyl violet is essential to render the capsule stainable.

The following table illustrates the importance of the colloidal solution of carbol-gentian-violet and acids in rendering the capsule stainable:

Thin Smears of Peritoneal Exudate Containing Many Pneumococci Were Fixed in Various Ways and Treated by the Following Solutions:	Coccus	Capsule	Albuminous Background
Sat. aq. sol. $HgCl_2$+carb.-gent.-violet	Deep purple	Invisible	Deep purple
Sat. aq. sol. $HgCl_2$+eosin	Unstained	Unstained	Light pink
Sat. aq. sol. $HgCl_2$+Gram iodin+eosin	Faintly pink	Unstained	Pink
Sat. aq. sol. $HgCl_2$+carb.-gent.-violet (from old al. sol. gent. violet)+Gram iodin+eosin	Deep purple	Pink	Light pink or purple
Sat. aq. sol. $HgCl_2$+carb.-gent.-violet (from new al. sol. gent. violet)+Gram iodin+eosin	Deep purple	Unstained	Light pink or purple
Heat+tannic or other acids+fresh carbol-gentian-violet +Gram iodin+eosin	Deep purple	Pink	Light pink
Tannic or other acids+eosin	Pink	Unstained	Light pink
Tannic-acid-gentian-violet+Gram iodin+eosin	Purple	Pink	Pink

As shown in the summary, acids increase the affinity of the capsule for eosin, under the conditions of the experiment, at the same time as they diminish the avidity with which the coccus retains the basic stain when treated by Gram's method. Another interesting observation is that if a smear is fixed in tannic-acid-gentian-violet, washed in water, treated with Gram's iodin, over-decolorized in alcohol and stained with eosin, capsule and coccus are stained deeply pink. If this smear is then treated a second time with gentian violet solution the affinity of the capsule for the basic dye is found to be greater than that of the coccus, which is exactly opposite to what it was in the beginning. A striking analogy to this behavior of pneumococci and their capsules has been observed

[1] *Boston Med. and Surg. Jour.*, 1910, 163, p. 791.

[2] *Die Theorie des Farbeprozesses*, 1910.

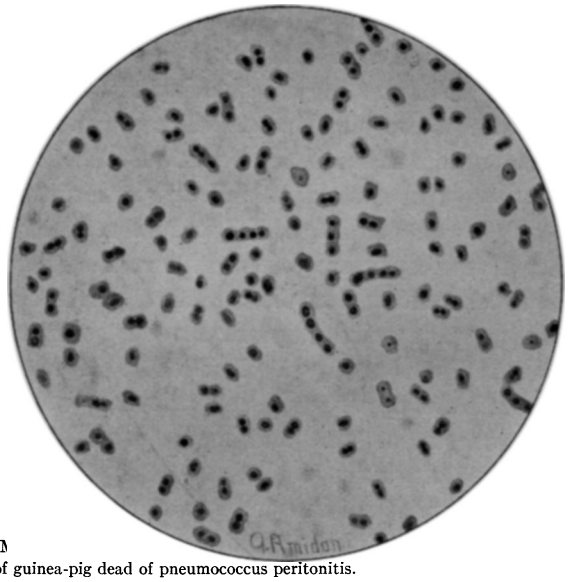

FIG. 1.—M from the peri
toneal cavity of guinea-pig dead of pneumococcus peritonitis.

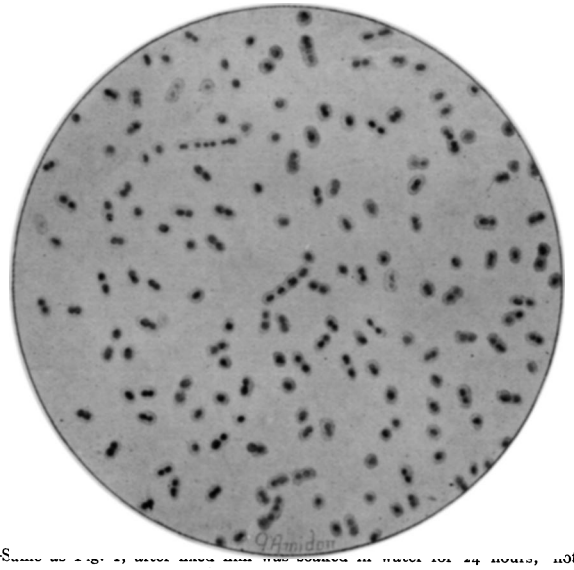

FIG. 2.—S ote the swollen
capsules.

when they undergo autolysis in NaCl or other solutions. Up to a certain point, usually at the end of from one to three days, depending upon the degree of virulence, the affinity of the coccus for basic dyes largely disappears, it becomes Gram-negative while the affinity of the capsule for eosin increases. At this point the capsules frequently appear empty. Gradually, as disintegration continues, the affinity for eosin also disappears. These observations with the fact that capsules are easily demonstrated after repeated washings in water indicate that the capsule of pneumococcus is an integral part of the organism and not merely a precipitation of albuminous material surrounding it. But that it plays a determining rôle in virulence is unlikely, because encapsulated pneumococci may be susceptible to phagocytosis and at the same time possess no noteworthy degree of virulence.

In this connection it is of interest to point out that when pneumococci have lost their affinity for basic dyes, the result of autolysis, and have become Gram-negative and eosin staining, they seem to have lost a large part of their toxic property. When injected subcutaneously they produce a prompt rise in opsonin with no negative phase. They now absorb opsonin from serum and are relatively more susceptible to phagocytosis.

A comparison of the new method with Buerger's and other methods in a study of various strains of *Streptococcus pyogenes*, *Streptococcus mucosus*, and pneumococcus in both exudate and cultures brings out the interesting fact that what has been looked on as a capsule around the coccus in former methods is only a retraction zone of the albuminous film, the result of the fixing process. In exudates *Streptococcus pyogenes* usually contains no capsule although occasionally, as in otitis media, the cocci are surrounded by a capsule which is indistinguishable from that of the pneumococcus. In cultures, however, the method is of great value in differentiating these organisms. The streptococcus rarely forms capsules, the pneumococcus regularly, soon after isolation. But the differentiation of the *Streptococcus mucosus* from a highly virulent pneumococcus on morphological and tinctorial grounds alone, even by the aid of the new method, is not always possible. It is true that the capsule of the former, as Buerger points out, is

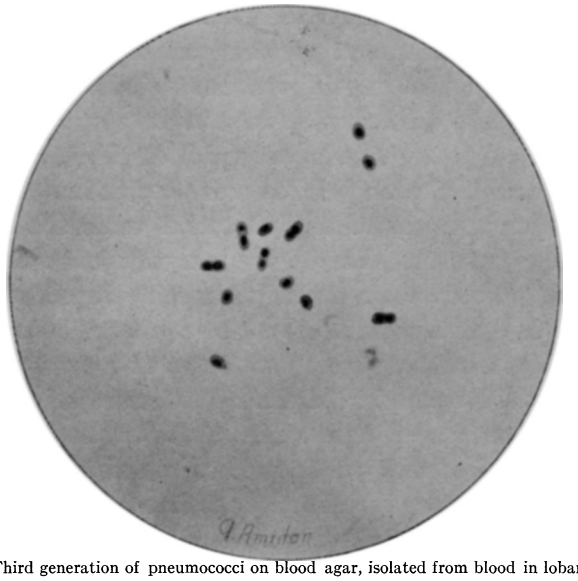

Fig. 3.—Third generation of pneumococci on blood agar, isolated from blood in lobar pneumonia soaked in water for 3 hours, and spread in serum.

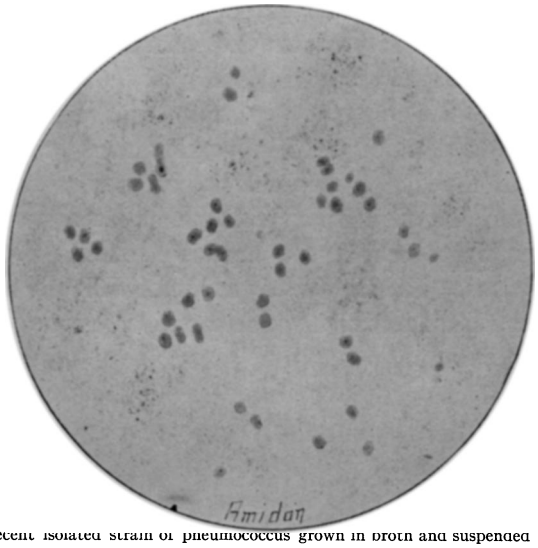

Fig. 4.—Recent isolated strain of pneumococcus grown in broth and suspended in NaCl solution at 37° C. for 24 hours. Note the many empty capsules.

usually more ragged and larger and the indentations not so regular about each diplococcus, yet pneumococci of high virulence, as Wadsworth has found, at times present similar pictures. The following facts seem to show that what in the past was taken for a solution of the capsule, when water is added after the smear was fixed on the slide, is probably simply an alteration in surface tension so that the capsule fails to take the stain under the conditions. Capsules of pneumococcus fixed by various ways and stained by the new method take the stain deeply after being "soaked" in water for several days to a week at 4° C., even after the center of the coccus has apparently disappeared. Beautifully stained capsules are given by this method when fixed (heat, chemicals) smears are washed in water for 2–24 hours and then covered with serum in a thin film and treated with tannic acid. If no serum or tannic acid is applied the capsule appears to have been dissolved, the area occupied by it being perfectly clear. During the process of autolysis in NaCl solution at 37° C., the capsule is the last to disintegrate. Welch noted lysis of pneumococci within their capsules in the exudate of resolving pneumonic lungs. I have found similar, often striking, pictures of empty capsules and cocci in all stages of disintegration inside of well-preserved capsules in pericardial, peritoneal, and pleuritic exudates, in smears of pneumonic lungs and sputum, in otitis media and mastoiditis.

Conclusions.—Contrary to the generally accepted view the capsule of pneumococci and allied organisms is not difficult to preserve nor is it readily soluble in water; and to stain it is a problem of rendering it stainable rather than one of preservation. The reactions which render the capsule stainable appear to be colloidal reactions. The capsule of pneumococci, while more stable than has been believed, is not a necessary factor in order to make pneumococci virulent.

PLATE 1.

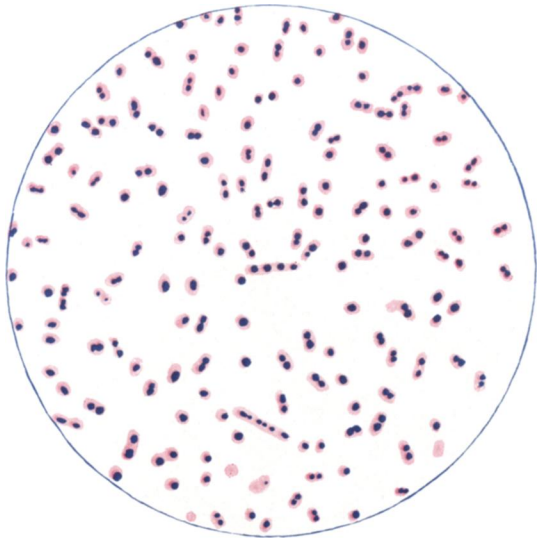

FIG. 1.—Same as Fig. 2 in text. The fixed film was soaked in water for 24 hours. See the swollen capsules

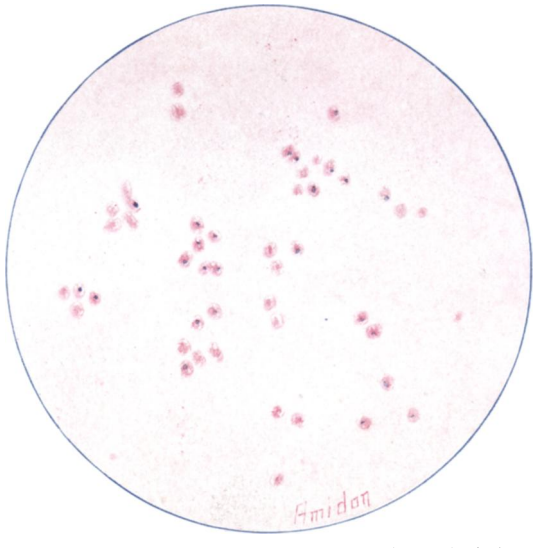

FIG. 2.—Same as Fig. 4 in text. Pneumococcus suspended in NaCl solution at 37 c. for 24 hours. Note the many empty capsules.

The smears used for the illustrations were fixed in saturated water solution of $HgCl_2$ and stained with carbol-gentian-violet, which was made from an old stock bottle of saturated alcoholic-gentian-violet, and counterstained with saturated solu-